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THE IDENTIFICATION AND MECHANISM OF FORMATION OF CRYSTALS FOUND IN THE CULTURE MEDIUM OF A MARINE BACTERIUM

A THESIS

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THE IDENTIFICATION AND MECHANISM OF FORMATION OF CRYSTALS FOUND IN THE CULTURE MEDIUM OF A MARINE BACTERIUM

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Chairman

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TABLE OF CONTENTS

LIST OF FIGURES

SUMMARY

The objectives of this research were to determine the structure of crystals which formed in the growth medium of the marine bacterium (coded 313A), to elucidate the mechanism of formation of these crystals, and to taxonomically classify the marine bacterium according to the scheme of Shewan, Hobbs, and Hodgkiss[22].

Based on the research reported in this text, the following conclusions were reached: The crystals are a hydrated form of magnesium ammonium phosphate. These crystals form as a result of the release of ammonia into the growth medium from the metabolism of nitrogenous substrates. The marine bacterium (313A) is tentatively classified to the genus Aeromonas.

CHAPTER I

INTRODUCTION

In the process of isolating bacteria from Antarctic marine sediment samples, it was observed that crystals formed in the maintenance medium of several isolates. These crystals appeared microscopically (50x) as transparent prisms which were insoluble in water and in sodium hydroxide but which were soluble in hydrochloric acid. Since the appearance of these crystals was quite different from that of granular amorphous type precipitates (such as calcium carbonate) which are sometimes seen in bacterial cultures, interest was aroused as to their identity.

After infrared spectral data had indicated that the crystals were magnesium ammonium phosphate, a literature search was made in order to investigate previous research in this area. The compound $MgNH_4PO_4 \cdot 6H_2O$ is classified by Dana's System of Mineralogy[5] as struvite, a mineral which sometimes occurs naturally. In nature, struvite has been found in organically rich deposits such as guano[5] or dung,[5] but very few reports of such struvite deposits have been published. Struvite has also been found in canned seafoods[8,11,15] and in cultures of various species of bacteria[1,9,20,21]. To my knowledge, however, the research reported here is the first in which magnesium ammonium phosphate has been isolated in connection with any species of marine bacterium, as well as the first time infrared spectroscopy (IR) has been utilized for magnesium ammonium phosphate identification. The mechanism of crystal formation by this

bacterium was studied in comparison with the mechanisms proposed by other workers for non-marine bacterial species which form the same crystals. Tests were then performed to taxonomically classify this particular marine organism to the genus level.

According to Beavon and Heatley,[1] Robinson[20] suggested in 1889 that ammonia produced by microorganisms from nitrogenous material combined with magnesium and phosphate already present in the culture medium to form magnesium ammonium phosphate. Since then, scattered reports have been published concerning the presence of struvite in bacterial cultures.

In 1927, Huddleson and Winter[9] reported the formation of magnesium ammonium phosphate in aerobic cultures of Brucella abortus and Brucella melitensis. These workers concluded that ammonia, formed by the organisms from the decomposition of amino acids, combined with magnesium phosphate, resulting in the precipitation of $MgNH_4PO_4 \cdot 6H_2O$. It was reported that these same crystals could be produced in the absence of bacterial growth in less than twenty-four hours by exposing the growth medium to ammonium hydroxide. The medium consisted of beef liver infusion, Bacto-peptone, sodium chloride, agar, tap water, and bromthymol blue. These data supported the earlier suggestion of Robinson.

In 1928, Scudder[21] reported the precipitation of magnesium ammonium phosphate crystals in cultures of Bacillus alcaligenes, Micrococcus catarrhalis, Corynebacterium diphtheriae and others. Crystal identification was based on a positive phosphate test and optical measurements (e.g, indices of refraction, optic axial angle, and dispersion). The basic medium used in these experiments had a beef heart hormone base with

0.5 percent agar, 1.0 percent peptone, and bromthymol blue. Species which produced crystals most readily in the basic medium produced minute glistening crystals when grown on a simple medium containing K_2HPO_4, peptone, and agar. Larger crystals of a feathery appearance were observed when these species were grown in the meat infusion hormone medium. It was also reported that when the basal medium was adjusted to alkalinity without additional ammonium ions no crystals formed.

In 1962, Beavon and Heatley[1] reported the occurrence of struvite in the culture media of Staphylococcus aureus, Pseudomonas pyocyanea, Escherichia coli and others. The crystals were identified by comparison with known struvite samples using the technique of X-ray crystallography. These workers, like Huddleson and Winter, reported that large numbers of crystals formed within a few minutes when the medium was exposed to ammonia. However, mere alkalinity, in the absence of additional ammonium ions, led to very scanty or no crystal formation.

CHAPTER II

METHODS AND MATERIALS

Source of Organism Used

The organism coded 313A used in this study was isolated on sulfate reducing medium[12] from the surface portion of a core sample of marine sediment taken at 61° 21'S and 96° 1'W at a depth of 4454 meters. The surface water temperature was 5 C and the salinity of the ocean water was 34.7 ppm. The samples were collected by Dr. Nancy W. Walls and Mrs. Dorothy E. DeFoor in April and May 1966 during Cruise Number 23 of the U.S.N.S. "Eltanin" in the Antarctic Ocean. Primary isolation of the organism was done by Mr. Ekkehart Gasper, Georgia Institute of Technology.

Morphology and Gram Reaction

A 30 hour Colwell and Quigley[18] broth culture preparation of the organism grown at 12 C was examined microscopically, using 1000x magnification, for morphology of stained cells and Gram reaction.

Motility

Motility was determined by stab inoculation into a 0.5 percent Colwell and Quigley agar medium, and by microscopic examination of a hanging drop preparation of the organism.

Leifson's Flagella Staining Method[14]

A 30 hour Colwell and Quigley broth culture (10 ml) grown at 12 C

was centrifuged at 2000g for thirty minutes at 4 C and the supernatant discarded. The bacterial cells were resuspended in two milliliters of Colwell and Quigley salt solution. A drop of this suspension was placed on a tilted, acid-cleaned, slide and allowed to run down the slide. The resulting smear was allowed to air dry. Approximately one milliliter of the staining solution (consisting of equal parts of 1.2 percent basic fuchsin in 95 percent ethanol, 1.2 percent tannic acid in distilled water, and 1.5 percent sodium chloride in distilled water) was then added to the area of the smear. The time required for staining the flagella was approximately eight minutes. After staining, the slide was washed quickly with tap water and carefully blotted.

Oxygen Requirement

The organism was isolated, maintained, and tested under aerobic conditions. To determine its ability to grow under anaerobic conditions, the organism was streaked on agar plates of Colwell and Quigley medium and incubated at 12 C in a Brewer "Gaspak" anaerobic jar.*

Temperature Requirement

To determine temperature dependence, Colwell and Quigley broth cultures were incubated at 4 C, 12 C, 20 C, 25 C, 30 C, and 37 C, and comparative growth was ascertained at various time intervals by optical density measurements with a Bausch and Lomb Spectronic 20 Colorimeter set at 600 mμ. Unless otherwise stated all cultures in the following text were grown at 12 C.

* Available from Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Maryland 21204.

Sodium Chloride Requirement

The salinity requirement of the bacterium was investigated by observing its growth in concentrations of sodium chloride from 0.0 to 3.0 percent in a basal medium: 1.0 percent proteose peptone, 0.3 percent yeast extract, and 1.5 percent Bacto-agar.

Hugh and Leifson Fermentation Test[10]

The medium used was composed of the following:

Trypticase (BBL-carbohydrate free).....	0.2 percent
NaCl.....	0.5 percent
K_2HPO_4.....	0.03 percent
Agar.....	0.3 percent
Bromthymol blue.....	0.003 percent
Glucose.....	1.0 percent

(Final pH 7.1)

A stab inoculation was made into each of two tubes of Hugh and Leifson medium from a 30 hour Colwell and Quigley broth culture. The medium in one tube was then overlaid with sterile mineral oil and both tubes incubated at 12 C.

Vibrostatic Effect of O/129[23]

Colwell and Quigley agar plates were inoculated with 0.3 ml of a 24 hour culture. After the inoculum was spread over the plate by swirling, a BBL Sensi-Disc saturated with a 0.1 percent solution of 2,4-diamino-6,7-diisopropyl pteridine (O/129) in acetone was placed on the agar surface. The plate was incubated for three days at 12 C and observed for growth around the disc.

Kovacs' Oxidase Test[13]

A small piece of filter paper was wet with 2-3 drops of a 1.0 percent solution of tetramethyl paraphenylenediamine dihydrochloride. A loop of culture from a 48 hour colony grown on Colwell and Quigley agar medium was quickly smeared over the saturated filter paper. A positive test was recorded if the transferred material turned purple within 5-10 seconds.

Media

The basic medium used in this study was devised by Colwell and Quigley[18] as a maintenance medium for bacteria isolated from deep sea sediments. The formula for this medium is:

Proteose peptone (Difco)	1.0	percent
Yeast extract (BBL)	0.3	percent
Bacto-agar	1.5	percent
Sodium chloride	2.4	percent
Magnesium chloride	0.53	percent
Magnesium sulfate	0.70	percent
Potassium chloride	0.07	percent

The components dissolved in deionized water gave a pH of 6.7 without further adjustment. Semi-quantitative analyses of the proteose peptone and yeast extract used in this medium appear in Appendix I. The basic medium minus proteose peptone was used to separate crystal types and will be referred to as yeast extract medium.

A solid medium consisting of 1.0 percent proteose peptone, 0.3

percent yeast extract, 1.5 percent agar, and 3.2 percent sodium chloride with no additional magnesium salts was prepared in order to study the effect of a great reduction in the magnesium ion concentration.

The organism was also grown and observed on Bacto-Marine Agar. The nutritional ingredients of this medium are peptone and yeast extract, while the salts composition approximates that of sea water. The following is a list of the salts contained in 1000 milliliters of this medium:*

Ferric Citrate.....	0.1	g.
Sodium Chloride.....	19.45	g.
Magnesium Chloride.....	8.8	g.
Sodium Sulfate.....	3.24	g.
Calcium Chloride.....	1.8	g.
Potassium Chloride.....	0.55	g.
Sodium Bicarbonate.....	0.16	g.
Potassium Bromide.....	0.08	g.
Strontium Chloride.....	0.034	g.
Boric Acid.....	0.022	g.
Sodium Silicate.....	0.004	g.
Sodium Fluoride.....	0.0024	g.
Ammonium Nitrate.....	0.0016	g.
Disodium Phosphate.....	0.008	g.

Crystal Collection

Crystals were collected from broth cultures and agar plates. The broth cultures were vigorously shaken to disperse the sedimented

* Difco, Supplementary Literature, 1962.

bacteria and crystals throughout the medium. Because the crystals settled faster than the bacteria, the supernatant material could be decanted shortly after agitation to separate crystals from the bacterial cell mass. The crystals were twice washed with distilled water, shaken, and the solutions decanted as before. The crystals were then washed twice with ethanol, followed by two washings with ethyl ether, and placed in a 37 C incubator for 15 minutes to evaporate the remaining ether.

Crystals from agar plates were more difficult to obtain, since the amount of heat needed to melt the agar (100 C) altered the structure of the crystals. Crystals formed in highest concentration on the surface of the agar immediately underneath the bacterial growth, however, and could be scraped off with the bacteria, leaving most of the agar. These scrapings were emulsified in water solution by rapid mechanical stirring with a Teflon stir bar. After stirring, the crystals sedimented first and the bacteria, agar, and extraneous material could be decanted. The crystals were then washed and dried as previously described.

pH Determination

In solid media, pH changes were measured by incorporating indicators into the growth medium. The two indicators used were 0.003 percent bromthymol blue which changes from green to blue at pH 7.1, and 0.02 percent phenol red which changes from colorless to red at pH 8.2[16]. In liquid culture media, pH was measured with a Beckman Zeromatic Meter, with standard glass and calomel electrodes.

Studies of crystal formation in broth cultures were done under the following conditions: Twenty tissue culture flasks, each containing forty

milliliters of Colwell and Quigley broth medium, were each inoculated with 0.5 milliliter of a 30 hour broth culture of the test organism. The flasks were incubated at 12 C on their sides, this positioning making the depth of the medium approximately seven millimeters. At twenty-four hour intervals, duplicate flasks were removed and the pH of each culture measured. The cultures were then centrifuged at 20,000g for ten minutes at 4 C to remove most of the bacterial cells. The supernatant liquids were decanted, sterilized by filtration (0.45 micron Millipore Filter),* and aseptically transferred to identical sterile flasks. The sterile medium was incubated at 12 C and observed for spontaneous crystal formation.

Infrared Analysis Technique

One to two milligrams of the crystal specimen to be analyzed was mixed with 200 milligrams of infrared quality potassium bromide and ground to a fine powder using an agate mortar and pestle. This mixture was pressed in a die under vacuum until the salt sintered and a clear pellet was formed. An infrared spectrum in the range 2.4 to 14 microns was obtained from the sample with a model 221 Perkin-Elmer Recording Spectrophotometer. The peak at 6.238 microns obtained from a 0.07 mm polystyrene film was used to standardize each spectrum.

* Available from Millipore Corp., Bedford, Mass. 01730.

CHAPTER III

RESULTS

Taxonomic Identification of the Organism

Morphology and Gram Reaction

When grown in Colwell and Quigley broth the stained cells appeared microscopically as Gram negative short rods with rounded ends, occurring singly and in short chains.

Motility

After 48 hours growth at 12 C in the stab inoculation medium, growth was seen distal to the line of inoculation, indicating motility. The organisms were observed to move in straight lines on microscopic examination of the hanging drop preparation.

Flagella Stain

In preparations stained by Leifson's method, the organism was found to possess a single polar flagellum (Figure 1 c).

Oxygen Requirement

The organism grew anaerobically at 12 C, but crystal formation was reduced to the extent that only a few small prisms were observed after twelve days incubation.

Temperature Requirement

The organism grew at all temperatures tested between 4 C and 37 C. Very poor growth was observed at 37 C; good growth at 4 C. The optimum growth temperature was between 20 C and 25 C. The maximum optical density

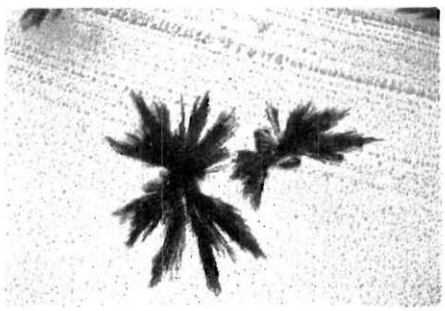

Figure 1a. Feathery Crystals.

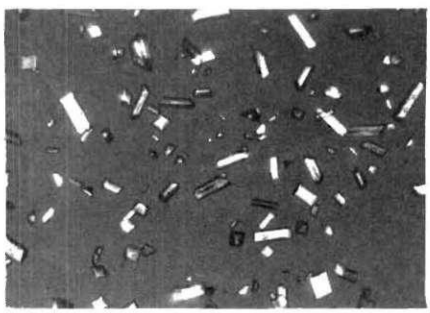

Figure 1b. Prismatic Crystals.

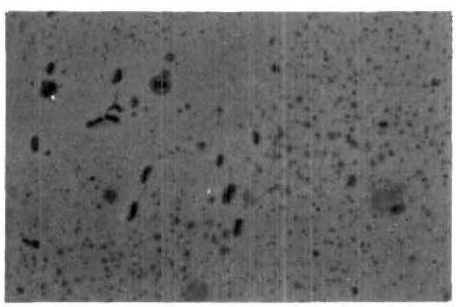

Figure 1c. Leifson's Flagella Stain Preparation of Test Organism.

reading for each temperature is given in the following table:

4 C (5 days).....0.500

12 C (50 hours).....0.300

20 C (50 hours).....0.660

25 C (50 hours).....0.700

30 C (50 hours).....0.105

37 C (50 hours).....0.078

Sodium Chloride Requirement

The following table indicates the amount of growth observed with various concentrations of sodium chloride added to the basal medium:

0.0 percent NaCl..... -

0.5 percent NaCl.....+++

1.0 percent NaCl.....++++

2.0 percent NaCl.....++++

3.0 percent NaCl.....++++

(- represents no growth; +++ represents good growth; ++++ represents abundant growth).

Hugh and Leifson Fermentation Test

Both the aerobic and anaerobic tubes of Hugh and Leifson medium became yellow (indicating acidic products) after eleven days incubation. Therefore the organism is considered to be a fermenter, according to the following scheme:

Glucose		
Aerobic (Open)	Anaerobic (Covered)	
-	-	I Nonoxidizers, Nonfermenters
A	-	II Oxidizers, Nonfermenters
A	A	III Fermenters

(- represents neutral or alkaline reaction; A represents acid reaction).

Vibriostatic Effect of 0/129

After three days incubation at 12 C, growth was observed next to the saturated Sensi-Disc. Sensitivity to the vibriostatic agent 0/129 would have prevented growth immediately surrounding the disc.

Kovacs' Oxidase Test

The bacterial mass smeared on the saturated filter paper turned purple within five seconds, indicating a positive test for oxidase.

Factors Influencing Crystal Formation

Effect of Inoculation Technique on Crystal Formation

When the organism was grown on the agar medium of Colwell and Quigley in 100 mm petri plates, two types of crystals were observed. If approximately two-thirds of the agar surface was inoculated with 0.2 milliliters of a 30 hour culture, many transparent prisms (Figure 1 b) would form at the interface of growth and agar surface within seven days. These crystals were of varying size, but only prisms were observed and these were located directly beneath the bacterial growth. Small prisms were especially concentrated along the margin of the diffuse colony. The second type of crystal had a feathery appearance (Figure 1 a) and was formed along with the prismatic type when a streak inoculation was made with a

wire loop across the agar surface. These feathery crystals were larger than the prisms, and in older cultures they sometimes formed in the agar distal to the bacterial growth. It was found that streak inoculation on yeast extract medium produced only the feathery crystals.

Effect of Magnesium Ion Concentration in the Medium

Reducing the magnesium ion concentration of the growth medium resulted in a marked decrease in crystal formation. Only very sparse crystals were observed after ten days inoculation. These crystals appeared as small multifaceted prisms.

Influence of pH on Crystal Formation

Only an estimate of pH could be made in agar plates, because incorporation of dye indicators into the growth medium during plate preparation was the sole means of pH measurement. When phenol red was used, the pH of the entire agar medium was observed to increase from 6.7 to at least 8.2 in 60 hours following a spread inoculation. The pH increased to a minimum of 8.2 within five days following a streak inoculation.

The increase of pH in forty milliliter Colwell and Quigley broth cultures as a function of time is shown in Figure 2. The pH increased from 6.7 at the time of inoculation (day 0) to a maximum of 8.0 on the sixth day and remained at approximately 8 through the eleventh day, after which no more flasks were tested for pH. The sterilized media were observed for a total of eighteen days from the start of the experiment and the following data collected: On the tenth day, crystals were observed in the several growing cultures which had not been sterilized. On the twelfth day crystals appeared in the sterilized medium which had been processed on the sixth day (from Figure 2 it can be seen that this culture

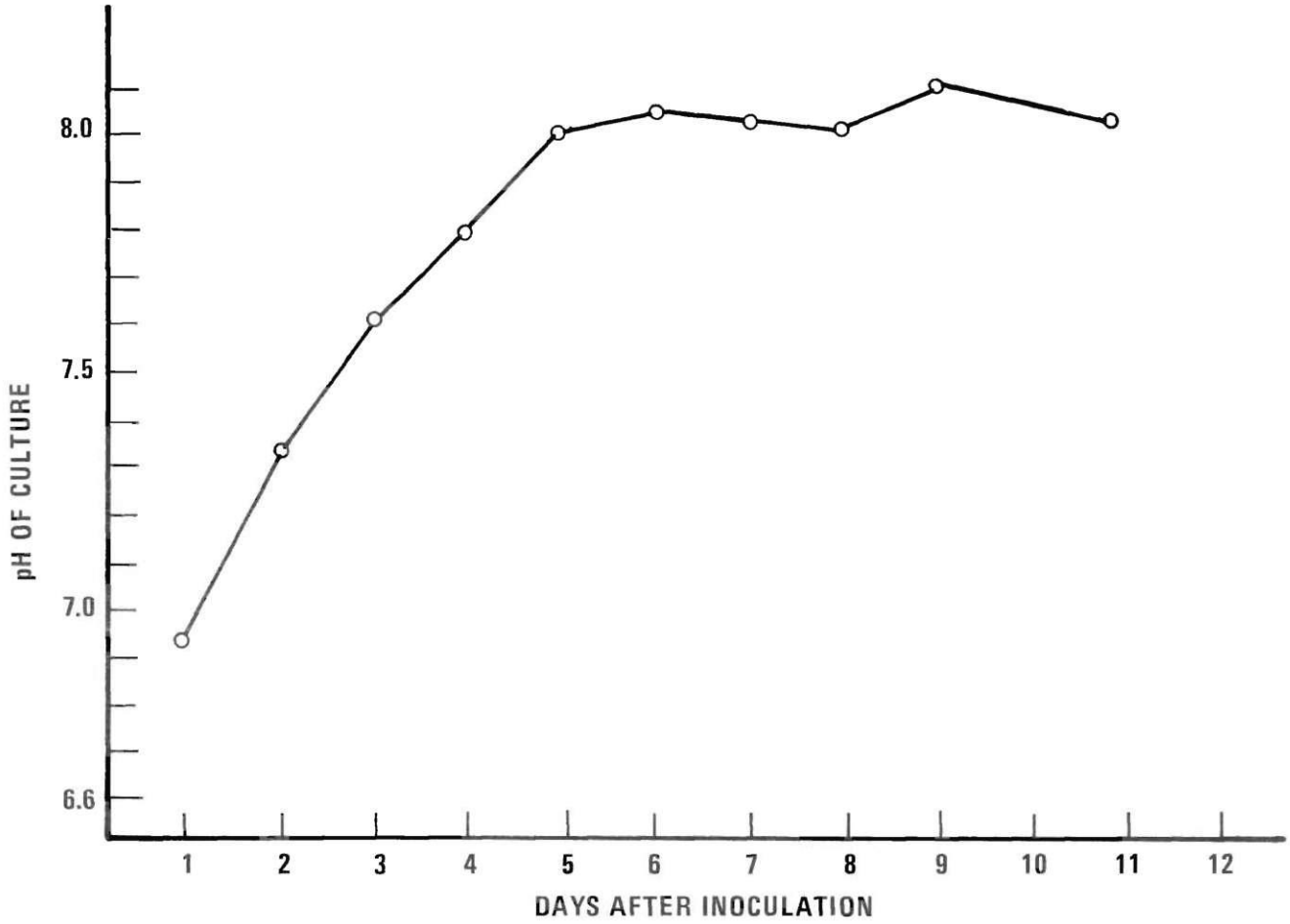

Figure 2. Graph of pH Values of Test Organism Cultures Versus Time (in days) After Inoculation.

was at pH 8.0 when sterilized). After eighteen days, crystals were observed in all the sterilized media with a pH $\geq$ 7.6.

An uninoculated flask of medium was adjusted to pH 8.0 with ammonium hydroxide and observed for crystal formation. After twenty-four hours many small crystals appeared in the medium.

The addition of ammonium hydroxide to uninoculated Colwell and Quigley agar medium produced vast quantities of crystals within several hours at 12 C; the addition of sodium hydroxide produced only an opalescence in the same type medium.

Crystal Formation on Marine Agar

Prismatic magnesium ammonium phosphate crystals were formed on Marine Agar after ten days incubation of inoculated plates at 12 C. Most of the crystals were observed under the bacterial growth; several prisms were seen in the agar distal to the bacterial growth. Two days after inoculation, before magnesium ammonium phosphate precipitated, tiny granular beads began to form. This material was presumed to be calcium carbonate because of its characteristic morphology[19] and the known presence of calcium in the medium.

Crystal Formation by Certain Other Marine Organisms

The following results were obtained when three marine bacterial species* (Flavobacterium marinotypicum, ATCC #19260, Achromobacter aquamarinus, ATCC #14400, and Pseudomonas fluorescens, ATCC #13042) were incubated at 12 C, and observed for crystal formation at intervals over a period of eighteen days. After ten days incubation, many large crystals

* American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md.

appeared exclusively under the bacterial growth of Achromabacter aquamarinus. After eighteen days incubation a very few of the large crystals had appeared under the growth in the Pseudomonad cultures. No crystals were observed under the growth of Flavobacterium marinotypicum throughout the eighteen days.

IR Spectral Identification of Crystals

The IR spectra of the feathery and prismatic crystal types are shown in Figure 3. A comparison of these spectra indicates that the two crystal types have the same chemical composition. By comparing these spectra with published spectra of inorganic compounds and with tables of peak intensities of inorganic ions[17] it was decided that the spectra in Figure 3 were due to the following ions: the broad peak at 9.95 microns results from phosphate ion, the peaks at 6.95 microns and 3.4 microns result from ammonium ion, and the peaks at 6.15 microns and 3.2 microns result from water of hydration. Considering the spectral data, and that a high concentration of magnesium was present in the growth medium, it seemed likely that the crystals were a hydrated form of magnesium ammonium phosphate. No spectrum of magnesium ammonium phosphate could be found in the literature, so a sample was prepared in the laboratory according to the procedure of Curtman[4]. An IR spectrum of this sample is shown in Figure 4 along with a spectrum of the prismatic crystals. These spectra indicate that both the feathery and prismatic crystals are hydrated magnesium ammonium phosphate.

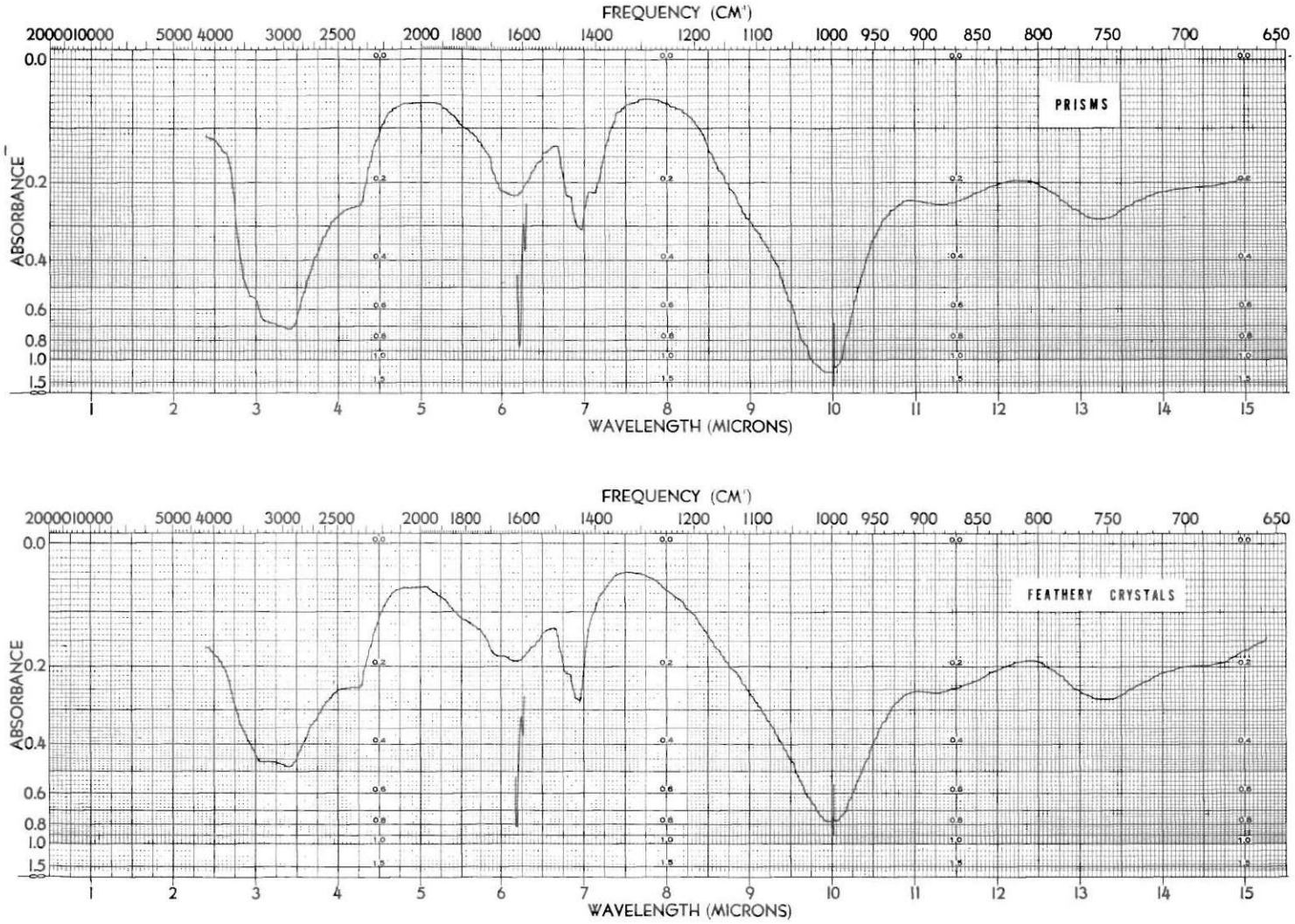

Figure 3. (Top) IR Spectrum of Prismatic Crystals Collected from Growth on Colwell and Quigley Agar Medium. (Bottom) IR Spectrum of Feathery Crystals Collected from Growth on Yeast Extract Agar Medium.

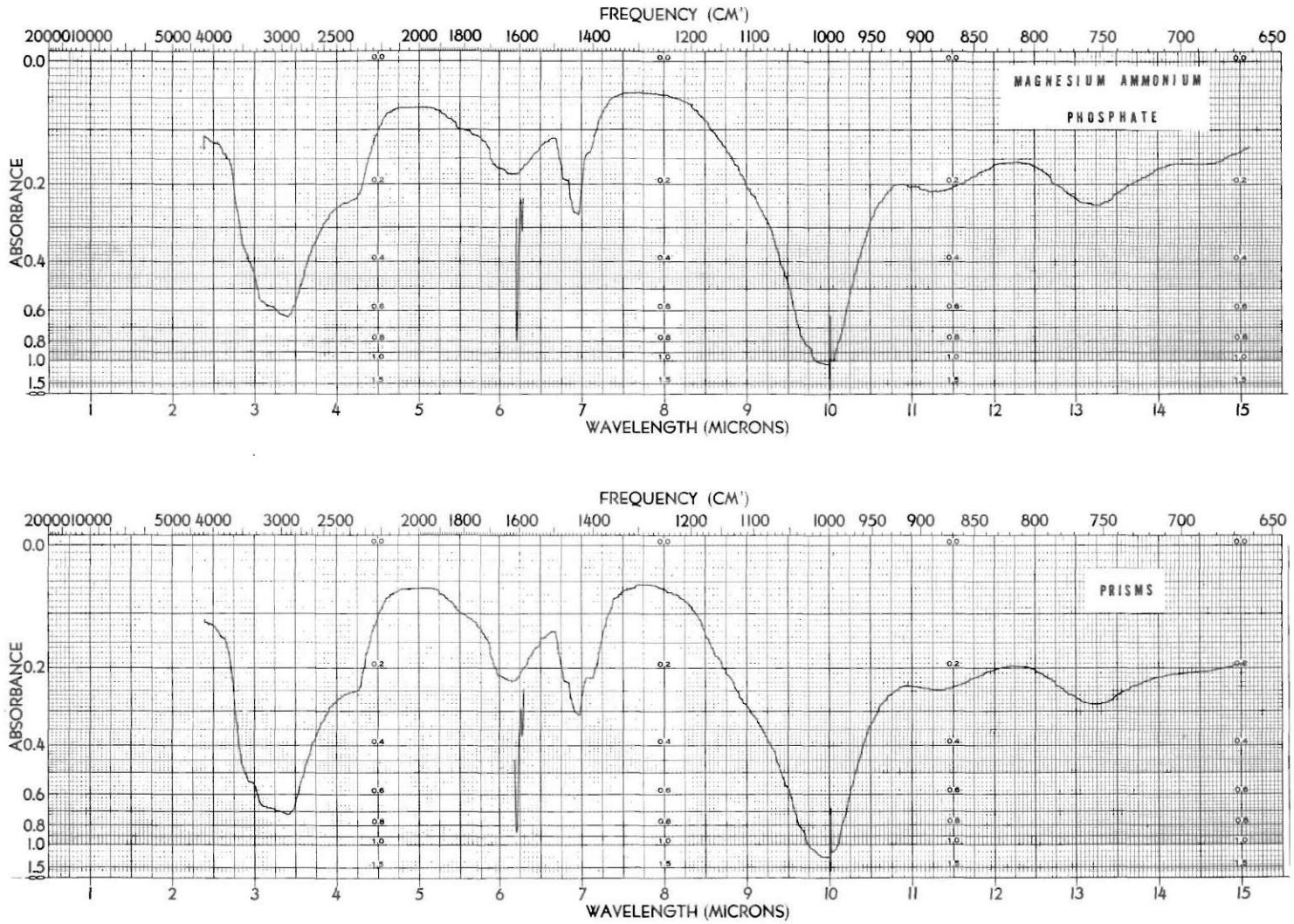

Figure 4. (Top) IR Spectrum of Known Magnesium Ammonium Phosphate Crystals Prepared in Laboratory. (Bottom) IR Spectrum of Prismatic Crystals Collected from Growth on Colwell and Quigley Agar Medium.

An IR spectrum of the crystals produced by adjusting Colwell and Quigley broth medium to pH 8.0 with ammonium hydroxide is shown in Figure 5. In the same figure, a spectrum is shown of the crystals collected from a bacterial broth culture at pH 8.0.

It was mentioned above (section on Crystal Collection) that the structure of these magnesium ammonium phosphate crystals was altered if they were heated to 100 C in an aqueous solution. A spectrum of the product obtained from heated crystals is shown in Figure 6. For comparison, a spectrum of magnesium phosphate ($Mg_3(PO_4)_2$) also appears in Figure 6 and a spectrum of $MgHPO_4$ is shown in Figure 7.

A spectrum of the prisms collected from the medium containing a reduced magnesium ion concentration is shown in Figure 7. A comparison of this spectrum with the spectra in figures 3, 4, and 5 indicates that these small multifaceted crystals are also a hydrated form of magnesium ammonium phosphate.

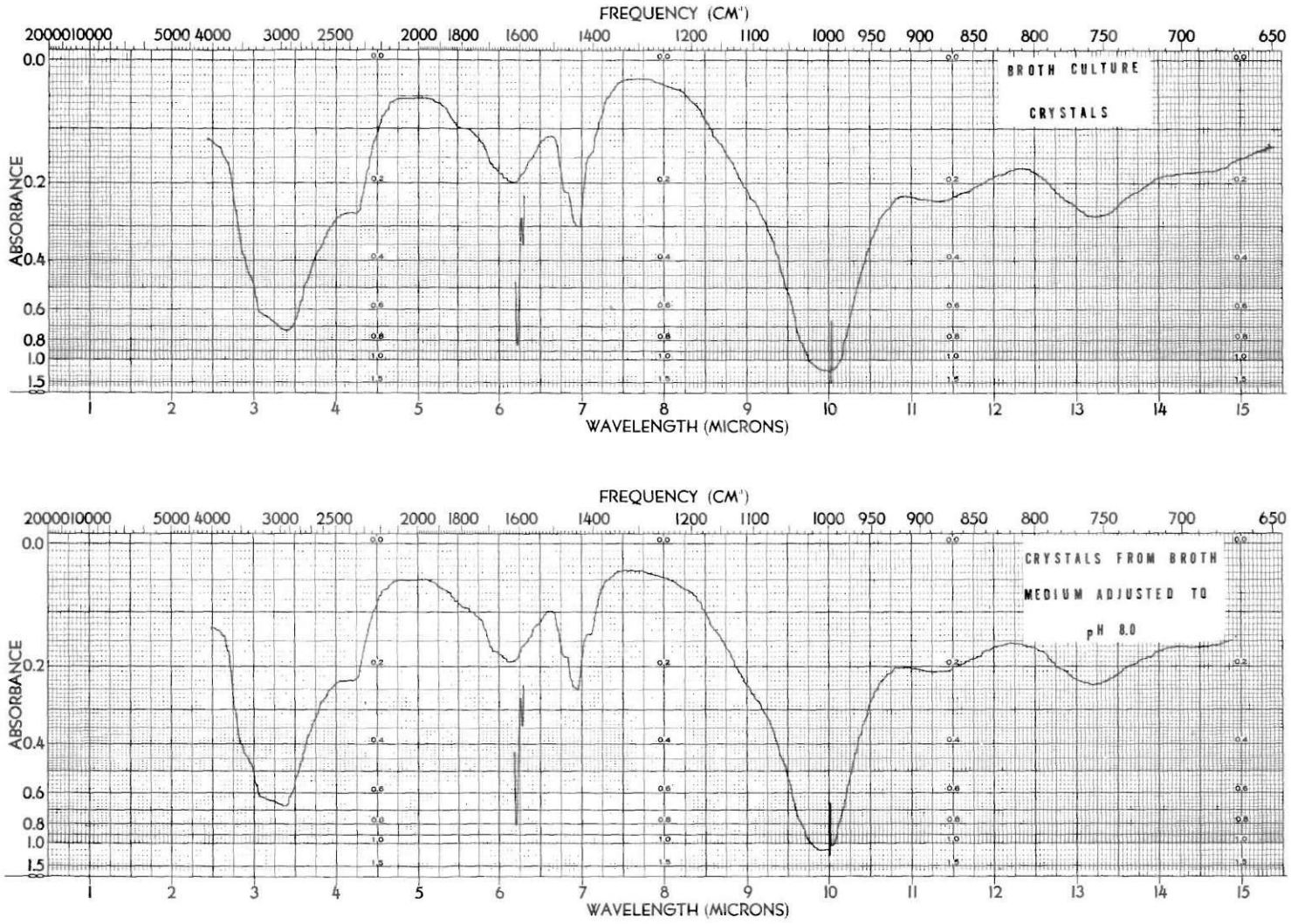

Figure 5. (Top) IR Spectrum of Crystals Collected After Growth in Colwell and Quigley Broth Medium. (Bottom) IR Spectrum of Crystals Collected from Sterile Colwell and Quigley Medium Adjusted to pH 8.0 with Ammonium Hydroxide.

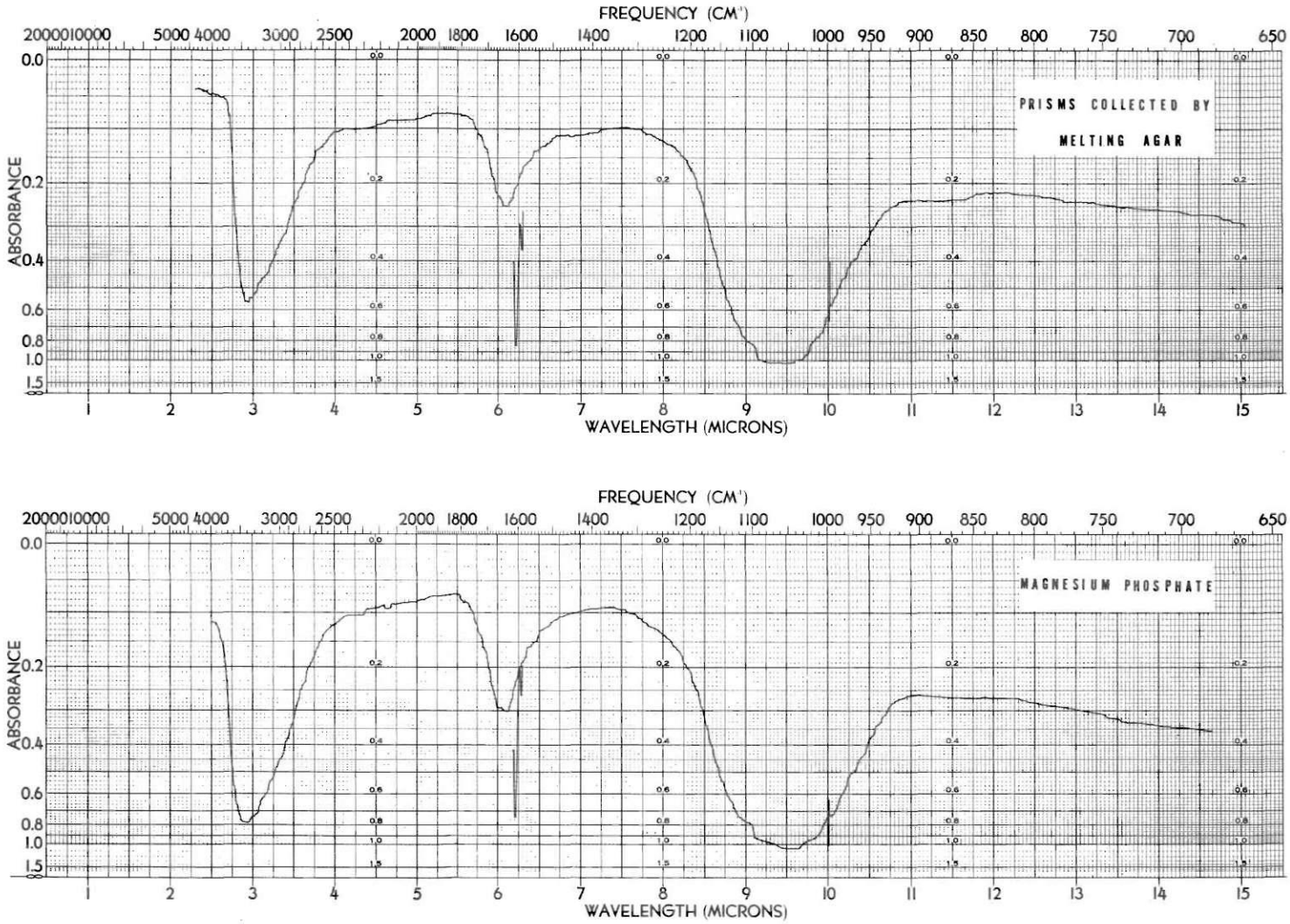

Figure 6. (Top) IR Spectrum of Crystals Collected from Colwell and Quigley Agar Medium Heated to 100 C. (Bottom) IR Spectrum of Magnesium Phosphate Crystals Prepared in Laboratory.

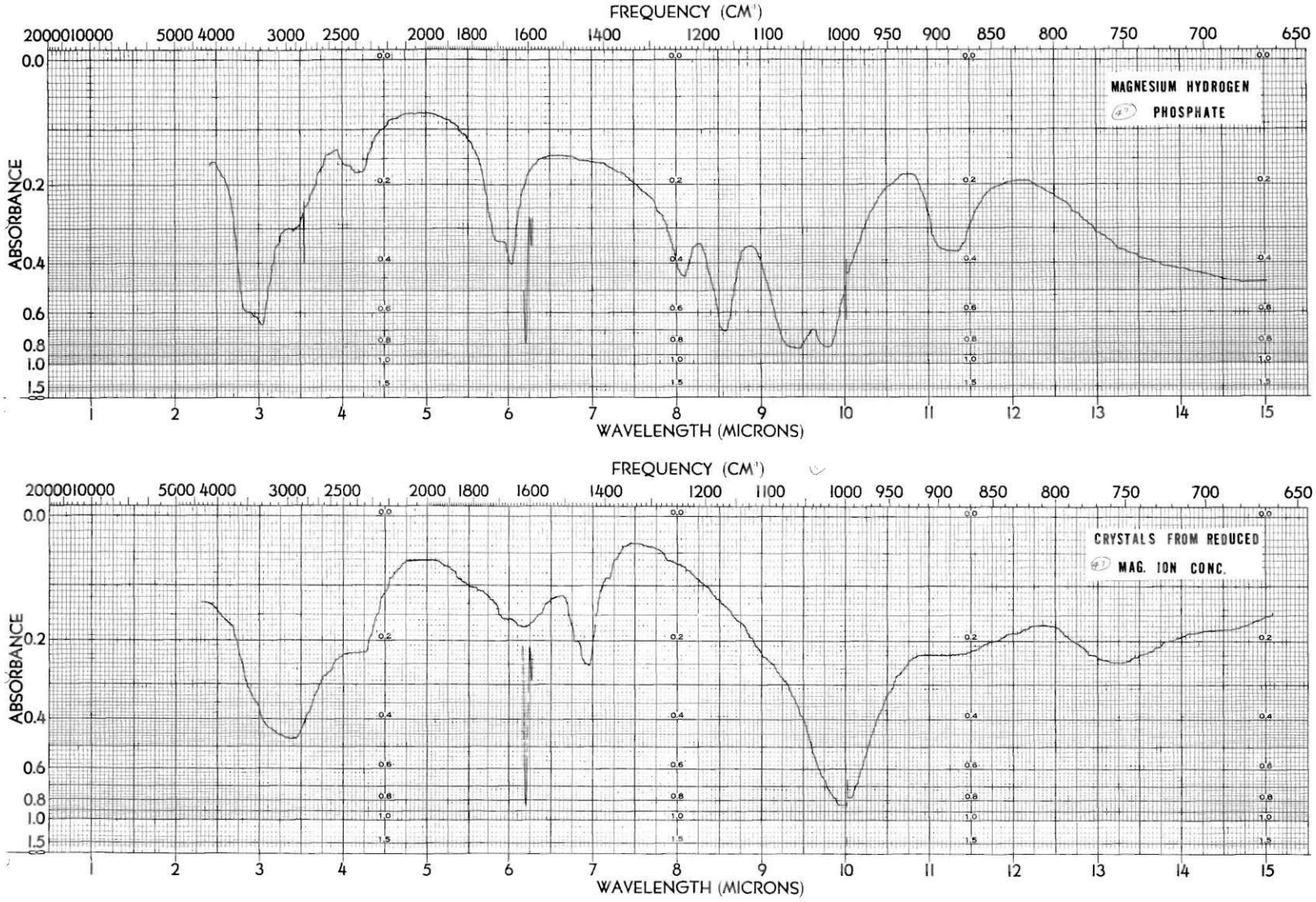

Figure 7. (Top) IR Spectrum of Magnesium Hydrogen Phosphate Crystals Prepared in Laboratory. (Bottom) IR Spectrum of Crystals Collected from Growth on Agar Medium Containing a Reduced Magnesium Ion Concentration.

CHAPTER IV

DISCUSSION

If the results obtained concerning bacterial classification are compared with the scheme of Shewan, Hobbs, and Hodgkiss,[22] shown in Figure 8, which classifies Gram negative rods, the organism (313A) may tentatively be classified to the genus level. The organism was placed within the group composed of Aeromonas, Pseudomonas, and Vibrio on the basis of the following characteristics: Gram negative, motile by means of a single polar flagellum, and oxidase positive. The genus Vibrio was eliminated because the organism was insensitive to the vibriostatic compound O/129. Similarly, the genus Pseudomonas was eliminated because the organism metabolized glucose fermentatively. Although the organism is tentatively assigned to the genus Aeromonas, more detailed study is required for a definite classification.

The infrared spectral data given in the results (Figure 3, 4, 5) are presented as evidence that both the feathery and prismatic crystals from solid and broth cultures are composed of magnesium ammonium phosphate. Removal of the ammonium ion from magnesium ammonium phosphate by heating this compound at 100 C results in a product that is indistinguishable from magnesium phosphate by infrared spectroscopy (Figure 6); thus it can be concluded that, upon heating in aqueous solution, magnesium ammonium phosphate decomposes to some hydrated form of $Mg_3(PO_4)_2$. The spectrum of

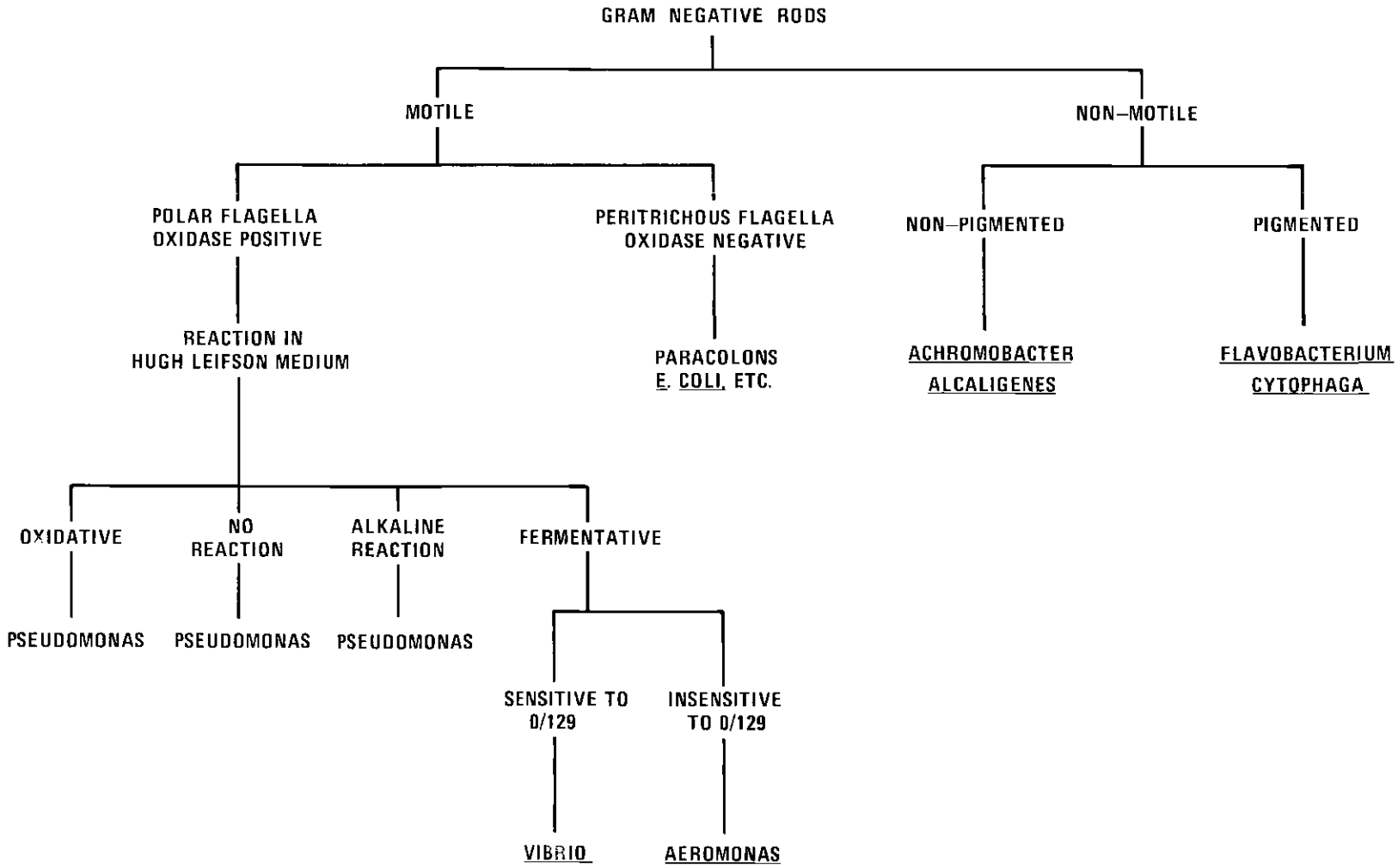

Figure 8. A Determinative Scheme for the Identification of Certain Genera of Gram-Negative Bacteria[22].

magnesium hydrogen phosphate ($MgHPO_4$), which appears in Figure 7, is easily distinguishable from either $MgNH_4PO_4$ or $Mg_3(PO_4)_2$ especially in the 8 to 10 micron region. A comparison of the spectra of these three compounds ($MgNH_4PO_4$, $MgHPO_4$, $Mg_3(PO_4)_2$) indicates that IR spectroscopy is a very acceptable technique for identification of magnesium ammonium phosphate, especially if it is necessary to distinguish it from its natural decomposition products $Mg_3(PO_4)_2$ and $MgHPO_4$.

The mechanism of formation of magnesium ammonium phosphate in cultures of the marine bacterium (13A) appears to be the same as those mechanisms proposed by other workers for non-marine bacterial species. Ammonia, produced as a by-product of bacterial metabolism, combines with magnesium and phosphate present in the medium to form magnesium ammonium phosphate. According to the data given in Figure 2, crystals form when broth cultures reach a pH $\geq$ 7.6, with or without the bacterial cells present. Since uninoculated medium adjusted to pH 8.0 with ammonium hydroxide forms identical crystals, but the addition of sodium hydroxide to agar medium of the same composition yields no crystals, it can be concluded that the production of ammonia is the only bacterial function necessary for magnesium ammonium phosphate crystal formation to take place in this medium.

As mentioned above, prisms which form in agar medium are observed only directly underneath the bacterial growth even though the pH of the medium is observed to be at least 8.2 throughout the plate. A possible

explanation for this is that ammonia which is produced by the organism reacts with water according to the following equilibrium:[4]

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

where $$\frac{[NH_4^+]\,[OH^-]}{[NH_3]} = K = 1.8 \times 10^{-5}$$

As ammonia is produced, a low concentration of ammonium ions is formed according to the above equilibrium. These ammonium ions are quickly complexed by the magnesium and phosphate ions present, thus forming the insoluble magnesium ammonium phosphate crystals. As the ammonium ions are complexed, the equilibrium is shifted to the right, and more ammonium and hydroxyl ions are formed. The hydroxyl ions diffuse throughout the medium, while the ammonium ions are complexed where they are produced. Hence, the entire medium would show a rise in pH but crystals would form only where the ammonia is produced.

Another question which arises from the results is: Why do two crystal types (feathery and prismatic) form if both are hydrated magnesium ammonium phosphate? The following statement concerning magnesium ammonium phosphate formed in human urine samples is found in Hawk's Physiological Chemistry[7]: "This substance may occur in the sediment in two forms, viz., prisms and the feathery type. The prismatic form predominates when the urine is made ammoniacal." The concentration of ammonia, therefore, seems to be the determining factor as to which type crystal forms, but at the present time no satisfactory explanation can be given as to why more ammonia is produced when a streak inoculation is used rather than a spread

inoculation, or yeast extract medium is used instead of the Colwell and Quigley medium.

The results of the experiments performed establish the fact that magnesium ammonium phosphate precipitates in the broth medium of Colwell and Quigley as a result of by-products produced by the organism during growth. A question of importance is: Will this magnesium ammonium phosphate be deposited in the marine environment? When the organism was grown on Marine Agar at 12 C, magnesium ammonium phosphate formed after 10 days incubation. The fact that the medium has essentially the same salts composition as sea water suggests that bacteria, given the right ecological conditions plus a proteinaceous substrate, could be responsible for the deposition of magnesium ammonium phosphate in the marine environment. However, reports of such marine deposition have not appeared in the literature. Cohen[3] reported that deposits of struvite ($MgNH_4PO_4 \cdot 6H_2O$) which once formed in or below guano deposits in Mono Lake, California, have now altered to $MgHPO_4 \cdot 3H_2O$ (newberyite)with the escape of ammonia from the environment. Frazier, et al.,[6] reported that magnesium ammonium phosphate undergoes hydrolytic alteration in water or in very dilute solutions of magnesium, ammonium, and phosphate ions; e.g., upon standing in water in an open container, struvite alters to $Mg_3PO_4 \cdot 8H_2O$ (bobierrite) within two months. It can therefore be concluded that, even if magnesium ammonium phosphate was produced in the marine environment by bacterial action, it would probably not remain stable long enough for large deposits to be formed and discovered.

CHAPTER V

CONCLUSIONS

Based on the research reported in the preceding text, the following conclusions are made:

According to the scheme of Shewan, Hobbs, and Hodgkiss, the test organism is classified as a member of the genus Aeromonas.

Hydrated magnesium ammonium phosphate is formed as a result of growth of the organism in Colwell and Quigley medium. The mechanism of crystal formation is very similar to those mechanisms proposed by others for non-marine species: Ammonia, formed as a by-product of metabolism, combines with magnesium and phosphate present in the medium to form magnesium ammonium phosphate.

IR spectroscopy was used as a new means of identification of magnesium ammonium phosphate. This technique was considered very satisfactory for the future identification of similar compounds formed by biological systems.

CHAPTER VI

RECOMMENDATIONS

Based on the results concerning crystal formation by the three ATCC marine bacterial species when grown in Colwell and Quigley agar medium, an interesting recommendation can be made for future research.

Bergey's Manual[2] states that Achromobacter aquamarinus and Flavobacterium marinotypicum produce ammonia from peptone. There is no mention of ammonia production from peptone by Pseudomonas fluorescens. It can be postulated that all bacterial species which produce ammonia from peptone do not form magnesium ammonium phosphate in this medium, and it may be that only the most proteolytic species produce enough ammonia to form magnesium ammonium phosphate. Therefore, the formation or non-formation of magnesium ammonium phosphate in this medium could be a guidepost to the proteolytic ability of a particular bacterial species. This information could be of some taxonomic value in the characterization of marine bacteria and the procedure certainly merits further investigation.

APPENDIX I

Proteose Peptone (Difco)*	Percent
Total Nitrogen	14.37
Primary Proteose N	0.60
Secondary Proteose N	4.03
Peptone N	9.74
Ammonia N	0.00
Free Amino N (Van Slyke)	2.66
Amide N	0.94
Mono-amino N	7.61
Di-amino N	4.51
Tryptophane	0.51
Tyrosine	2.51
Cystine (Sullivan)	0.56
Organic Sulfur	0.60
Inorganic Sulfur	0.04
Phosphorus	0.47
Chlorine	3.95
Sodium	2.84
Potassium	0.70
Calcium	0.137
Magnesium	0.118
Manganese	0.0002

* Difco Manual, Ninth Ed., 1953

Proteose Peptone (Difco)	Percent
Iron	0.0056
Ash	9.61
Ether Soluble Extract	0.32

Yeast Extract (BBL)*

Nitrogen	Percent
Total	10.3
Amino	5.5
Amino Acids	**Percent**
Arginine	3.5
Cystine	1.6
Histidine	1.5
Isolencine	4.7
Lencine	6.4
Lysine	6.5
Methionine	2.0
Phenylalanine	3.5
Threonine	3.3
Tryptophane	1.0
Tyrosine	4.0
Valine	4.8
Carbohydrate Percent	16.6

* Personal Communication from Baltimore Biological Laboratory.

Other	Percent
NaCl	0.5
Calcium	0.06
Iron	0.20
Potassium	3.4
Magnesium	0.07
Phosphorus	1.16

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